Original Article

Sexual Development

Sex Dev 2011;5:33–47
DOI: 10.1159/000322579

Accepted: September 22, 2010
by M. Schmid
Published online: December 18, 2010

Elevated *amh* Gene Expression in the Brain of Male Tilapia *(Oreochromis niloticus)* during Testis Differentiation

S. Poonlaphdecha[a] E. Pepey[a] S.-H. Huang[a] M. Canonne[a] L. Soler[a]
S. Mortaji[a] S. Morand[b] F. Pfennig[c] C. Mélard[d] J.F. Baroiller[a] H. D'Cotta[a]

[a]Aquaculture Research Unit, CIRAD-PERSYST, [b]Institut des Sciences de l'Evolution, CNRS, Université Montpellier 2, Montpellier, France; [c]Institute of Zoology, TU Dresden, Dresden, Germany; [d]University of Liège, CEFRA, Liège, Belgium

Key Words
amh/Amh · Aromatase · Development · Sex differentiation · Sex dimorphism · Tilapia

Abstract
Anti-müllerian hormone (AMH) is expressed in male embryos and represses development of müllerian ducts during testis differentiation in mammals, birds and reptiles. *Amh* orthologues have been identified in teleosts despite them lacking müllerian ducts. Previously we found sexually dimorphic aromatase activity in tilapia brains before ovarian differentiation. This prompted us to search for further dimorphisms in tilapia brains during sex differentiation and see whether *amh* is expressed. We cloned the tilapia *amh* gene and found that it contains 7 exons but no spliced forms. The putative protein presents highest homologies with Amh proteins of pejerrey and medaka as compared to other Perciformes. We analysed *amh* expression in adult tissues and found elevated levels in testes, ovary and brain. *Amh* expression was dimorphic with higher levels in XY male brains at 10–15 dpf, when the gonads were still undifferentiated and gonadal *amh* was not dimorphic. Male brains had 2.7-fold higher *amh* expression than gonads. Thereafter, *amh* levels decreased in the brain while they were up-regulated in differentiating testes. Our study indicates that *amh* is transcribed in male brains already at 10 dpf, suggesting that sexual differentiation may be occurring earlier in tilapia brain than in gonads.

Anti-müllerian hormone also known as the müllerian inhibiting substance (AMH/MIS) plays a primordial role in the development of the urogenital system of mammals, birds and reptiles during sex differentiation. Initially the mammalian embryos of both sexes develop 2 pairs of genital ducts: the wolffian and the müllerian ducts [Josso et al., 2001]. In male embryos AMH represses the development of the müllerian ducts which would otherwise develop into the fallopian tubes, uterus, and upper vagina in female embryos [Munsterberg and Lovell-Badge, 1991]. AMH is male-specific in embryos and produced by Sertoli cells at the onset of testis differentiation [Morrish and Sinclair, 2002]. AMH also exerts a negative control on estrogen production since foetal ovaries treated in vitro with AMH showed a sex reversal of their steroidogenic pathway, producing testosterone instead of estradiol [Vigier et al., 1989]. This steroid modification was due to the

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1661–5425/11/0000–0000$38.00/0

Accessible online at:
www.karger.com/sxd

Helena D'Cotta
Aquaculture Research Unit, CIRAD-PERSYST
Campus International de Baillarguet
FR–34398 Montpellier Cedex 5 (France)
Tel. +33 46759 3951, Fax +33 46759 3825, E-Mail dcotta@cirad.fr

suppression of aromatase activity, the estrogen-synthesizing enzyme, as well as the down-regulation of luteinising hormone (LH) receptors [Vigier et al., 1989; Di Clemente et al., 1992]. In foetal rat Leydig and Sertoli cells, aromatase activity and aromatase *Cyp19a* gene expression were also suppressed by AMH [Rouiller-Fabre et al., 1998]. Furthermore, AMH was capable of inhibiting testosterone production of foetal Leydig cells [Rouiller-Fabre et al., 1998].

In males AMH persists until the onset of puberty with an inhibitory effect on spermatogenesis in Leydig cells [Josso and Clemente, 2003]. *Amh* is also expressed in the granulosa cells of postnatal females playing a role in follicle development [Ueno et al., 1989; Durlinger et al., 2002]. AMH is a dimeric glycoprotein of the transforming growth factor-β (TGF-β) super-family [Rey et al., 2003] acting via a signalling pathway consisting of 2 types of receptors with only type II AMHR2 being specific [Teixeira et al., 1999; Josso et al., 2001]. SOX9 in conjunction with SF1, GATA4 and WT1 up-regulate *AMH* in male foetuses [De Santa Barbara et al., 1998; Arango et al., 1999; Tremblay and Viger, 1999].

Unlike mammals, *AMH* in birds and reptiles plays a role in the early testicular fate [Smith and Sinclair, 2004; Shoemaker and Crews, 2009]. *AMH* expression in chicken is detected in both differentiating males and females, and it precedes that of *SOX9* at early stages of sex differentiation [Oreal et al., 1998; Smith et al., 1999]. This indicates that *AMH* is not regulated by *SOX9* in chicken despite the finding of 2 putative *SOX* regulatory elements in the *AMH* promoter [Oreal et al., 1998]. In the red-eared slider turtle and the alligator, species with temperature sex determination, *AMH* expression is higher in the bipotential gonads at male-promoting temperatures [Western et al., 1999; Shoemaker et al., 2007]. Like in birds, the regulatory role of *SOX9* on *AMH* does not appear to be conserved in reptiles [Shoemaker and Crews, 2009].

Amh has also been found in various teleost species despite the absence of müllerian ducts in these fish. Müllerian ducts have only been described in basal ray-fin fish such as sturgeons and they do not degenerate [Wrobel, 2003]. *Amh* was initially expressed in both sexes and then became dimorphic, expressed strongly in the pre-Sertoli cells of testes, as sex differentiation progressed in the Japanese flounder [Yoshinaga et al., 2004], Nile tilapia [Ijiri et al., 2008] and rainbow trout [Vizziano et al., 2008]. It was also expressed at male-promoting temperatures in the pejerrey [Fernandino et al., 2008] and in the Japanese flounder [Kitano et al., 2007]. In zebrafish, *amh* expression was low in the undifferentiated gonad [von Hofsten et al., 2005] but increased strongly at the onset of the juvenile ovarian-to-testis differentiation [Rodríguez-Marí et al., 2005; Wang and Orban, 2007]. However, no sex-linked differences were observed for *amh* and *amhr2* in the medaka, the only species where the receptor has been cloned [Klüver et al., 2007]. Amh/Amhr2 signalling has recently been found in medaka to be implicated in the regulation of germ cell proliferation in both males and females [Morinaga et al., 2007; Shiraishi et al., 2008]. The role of *amh* in sex differentiation of teleosts still remains unclear. A hypothesis is that Amh could be an anti-aromatase factor as reported in mammals, regulating estrogen levels of future males. Although Amh levels are inversely expressed with respect to *cyp19a1a* expression at certain stages of sex differentiation in some species [Rodríguez-Marí et al., 2005; Wang and Orban, 2007; Fernandino et al., 2008; Ijiri et al., 2008; Vizziano et al., 2008], it is not clear whether *amh* down-regulates *cyp19a1a* expression. Treatments with tamoxifen and hormone supplement in the Japanese flounder suggested that estrogens promoted the up-regulation of *cyp19a1a* expression but also the suppression of *amh* [Kitano et al., 2007].

Sex steroids are known to induce sexual differentiation of various brain regions [Arnold et al., 2004]. Other effectors than testosterone appear to also act on brain sex differentiation even before gonadal factors [Dewing et al., 2003]. Recently the brain of adult mice was found to synthesize AMH and its receptor in motor neurons, mediating their survival in vitro [Wang et al., 2005]. In embryos, *Amh* expression was not detected in the brain but sex differences in the number of motor neurons were attributed to *Amh* and this was independent of androgen-induced dimorphism [Wang et al., 2009]. The authors also showed that *Amh* contributed to the sex differences in behaviour. These results and the fact that *Amhr2* was expressed in most neurons led Wang et al. [2009] to suggest that *Amh* is a broad regulator of neurons, and that it is 'one of the factors causing sex-linked biases and variability within male lineage'.

Brain aromatisation occurs in vertebrates, but its activity is particularly elevated in teleosts during reproduction [Diotel et al., 2010]. We found that tilapia brain had elevated aromatase enzyme activity which was sexually dimorphic before the onset of ovarian morphological differentiation [D'Cotta et al., 2001]. Moreover, temperature-masculinisation of XX tilapia suppressed both the gonadal *cyp19a1a* gene expression and the brain aromatase activity, suggesting that a tight regulation between

the gonads and the central nervous system may exist during sex differentiation in this species. We undertook this study to molecularly characterise *amh,* analyse whether *amh* is expressed in the brain and establish its temporal expression with regard to gonadal *amh* expression during tilapia brain development and the sex-differentiating process. Only partial regions of the tilapia *amh* have been cloned or studied previously [Shirak et al., 2006; Ijiri et al., 2008]; we therefore also cloned the tilapia gene and analysed its gene structure in order to see whether transcriptional differences exist in this species as already described in the European sea bass [Halm et al., 2007].

Materials and Methods

Animals and Samplings

Several genetic all-female XX and all-male XY progenies of Nile tilapia *Oreochromis niloticus* were produced. The all-female progenies were generated by mating XX males (11β-hydroxyandrostenedione treated) with XX females [D'Cotta et al., 2001]. The all-male progenies were sired by YY males mated with normal XX females. These YY males were identified through progeny testing of XY females (treated with 17α-ethinylestradiol) mated with normal XY males as mentioned in Baroiller and Jalabert [1989]. After artificial fertilisation, the eggs were incubated in McDonald bottles and then kept in rearing aquaria at 27°C at the Cirad facilities. After hatching (~4 days post fertilisation = dpf) the fish were kept under constant photoperiod (12:12 h) and fed ad libitum. The fish were sampled from 10 to 26 dpf, euthanized using lethal doses of Eugenol and the heads and both the gonads were dissected under a stereo microscope and kept in RNAlater (Ambion, UK). We also opted to sample trunks with gonads after removing the viscera, because it enabled us to sample many more progenies. At each sampling date a minimum of 50 gonads were pooled per progeny, and at least 20 heads or 20 trunks were pooled per progeny. Additional head samples were taken at 14 dpf from males and females and part of the heads were dissected to separate brains, gills and eyes for analysing *amh* expression. Sex ratios were determined by gonadal squash after 3 months of age using at least 100 individuals per progeny [D'Cotta et al., 2001]. In addition, all organs were dissected from both adult females and males to define *amh* tissue expressions. All work on animals was conducted according to the Cirad stipulations and to the French regulations on animal welfare.

Total RNA Extraction, DNase Treatment and Reverse Transcription

Total RNA was extracted from the different organs using the TRIzol reagent (Invitrogen, Cergy-Pontoise, France). Homogenisation of the organs was performed on a tissumizer MM301 (Retsch, Haan, Germany) using glass beads. The gonads were further lysed by several aspirations through a 1-ml syringe and a 17-gauge needle. The subsequent steps were essentially those described by the manufacturer. Quantification of RNA was assessed by spectrophotometry, BioWaveII (WPA, Cambridge, UK) and RNA integrity was evaluated on a 1% agarose gel. To remove possible genomic DNA, total RNA was treated with 0.5 U TURBO DNase (Ambion, Austin, Tex., USA) incubated at 37°C for 30 min, after which EDTA was added to a final concentration of 5 mM and the DNase was subsequently inactivated by heating at 75°C for 10 min. For the trunks, heads and adult tissue, 3 μg of DNase-treated total RNA was used to generate the first-strand cDNA, whereas for the gonads 1 μg of total RNA was used. RNA was first heated for 5 min at 65°C with 1 μl of oligo $(dT)_{12–18}$ primers (Invitrogen, Karlsruhe, Germany) and 1 μl of 10 mM dNTPs and then chilled on ice. The reverse transcription was performed with 4 μl of 5× first-strand buffer, 2 μl of 0.1 M DDT, 1 μl of RNaseOUT (Invitrogen) and 1 μl of SuperScript™ II reverse transcriptase (Invitrogen) and carried out at 42°C for 60 min. The reaction was inactivated by heating at 70°C for 15 min. The cDNA was diluted to 3 ng/μl (estimated on initial RNA amounts) and 15 ng/μl for pooled and adult organs respectively in real-time PCR and stored at –20°C prior to their use.

Cloning of the Nile Tilapia amh *Gene*

To isolate the Nile tilapia *amh* orthologous gene, we derived several primers from GenBank sequence DQ257619 predicted as *amh* from blue tilapia, *O. aureus*. With primers amh_F2: 5′-GCA AAC ATG TTG GGT CTG CTC G-3′ (located around the putative start codon) and amh_R: 5′-AAA TGC TGG GGT AAA GTA GCA GAA GAG-3′ (located shortly after the putative stop codon) we were able to amplify a 1,585-bp fragment from testis cDNA. The PCR product was ligated into the pCR2.1 vector by TA cloning (Invitrogen). Sequencing revealed high similarity of the cloned PCR product with known teleost *amh* sequences. For isolation of the genomic *amh* sequence from *O. niloticus* the forward primer amh_F: 5′-GCA CGC GCA GAC ACT GC-3′ (located directly before the putative start codon) and the reverse primer amh_R as above were used. The resulting 3,142-bp PCR product was cloned into pCR2.1 vector. PCR conditions for both cloning procedures were 6 μl 5× reaction buffer, 3 μl of 25 mM $MgCl_2$, 0.6 μl dNTP mix (10 mM each), 1 U GoTaq (Promega, Mannheim, Germany) in a total volume of 30 μl. Each primer was used at a final concentration of 500 nM using as template either cDNA obtained from 50 ng of initial total RNA or 100 ng of genomic DNA from tilapia males. Cycling parameters were: 95°C for 3 min, followed by 35 cycles of amplification at 95°C for 30 s, 62°C for 30 s and 72°C for 180 s. The sequence of the Nile tilapia *amh* gene was deposited in GenBank under accession number EF512167.

Protein Alignment and Phylogenetic Analysis

Protein alignment and phylogenetic analysis were done using the pipeline phylogeny.fr [Dereeper et al., 2008] from the LIRMM (Laboratoire d'Informatique, de Robotique et de Microélectronique de Montpellier). This pipeline provides different programs for alignment, curation, phylogeny and tree rendering. Protein alignment was generated with multiple sequence comparison by log-expectation (MUSCLE version 3.5) [Edgar, 2004]. After alignment, ambiguous regions were removed with Gblocks (v0.91b). Phylogenies were determined using the Neighbour-Joining Method from the PHYLIP package (v3.67) [Saitou and Nei, 1987] with a bootstrap value of 1,000. Graphical representation and edition of the phylogenetic tree were performed with TreeDyn (v198.3) [Chevenet et al., 2006]. Protein accession numbers used for phylogenetic Amh analysis were: Nile tilapia *Oreochromis niloticus* (EF512167); blue tilapia *Oreochromis aureus* (EU219911); European sea bass *Dicentrarchus labrax* (AM232701); black porgy

Acanthopagrus schlegelii (GU256046); pufferfish *Takifugu rubripes* (ENSTRUG00000017845); tetraodon *Tetraodon nigroviridis* (ENSTNIP00000000587); Japanese flounder *Paralichthys olivaceus* (AB166791); medaka *Oryzias latipes* (ENSORLP00000006358); pejerrey *Odontesthes bonariensis* (AY763406); stickleback *Gasterosteus aculeatus* (ENSGACP00000016697); Atlantic salmon *Salmo salar* (AY722411); zebrafish *Danio rerio* (AY677080); squalius *Squalius pyrenaicus* (EU136185); human *Homo sapiens* (NM_000479) and mouse *Mus musculus* (NM_007445).

Quantitative Real-Time PCR Analysis

Quantitative real-time PCR was performed using a Stratagene MX3000P QPCR system (Stratagene, La Jolla, Calif., USA). Primers were designed using Beacon Designer 6 software or manually and then analysed with the IDT SciTools OligoAnalyzer 3.1 Software (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/). The *amh* primers used for quantitative real-time PCR were the following: amh_F1577: 5′-AAGCAGCGCAAACATTAACA-3′ and amh_R1744: 5′-GTTCCAGTCCACAACCTCCA-3′ which gave a product of 169 bp and the primers for *ef1* were ef1_F832: 5′-TGTTGAGACTGGTATCCTGAAGCC-3′; ef1_R1072: 5′-GATGATGACCTGAGCGTTGAAGC-3′ giving a product of 240 bp in size. All primers had a T_m of 60°C and were synthesised by Eurogentec (Belgium). The real-time PCR reactions were carried out in 20 μl volume containing 2 μl of 10× reaction buffer, 1.4 μl of 50 mM $MgCl_2$, 0.8 μl of dNTP mix, and 0.6 μl of the SYBR Green kit (Eurogentec, Seraing, Belgium) following the manufacturer's instruction using a final concentration of 450 nM for each primer with 9 and 45 ng of initial total RNA of pooled and adult organs, respectively. Duplicates were run for each sample. Cycling parameters were: 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, and then by a dissociation at 95°C for 1 min, 55°C for 30 s and 95°C for 30 s. The QPCR reaction without the template (None Template Control = NTC) was systematically included. Primer specificity was verified by a final dissociation curve in which an only peak was obtained. In addition, the PCR product was sequenced. The cycle threshold (Ct) was calculated for the average of 2 duplicate analyses performed for each individual sample. For each gene the amplification efficiency (E) was calculated from gene-specific standard curves performed on a serially diluted PCR product and calculated according to the equation $E = 10^{(-1/slope)}$. Expression amounts were calculated from a modified ΔCt method which takes into consideration different PCR efficiencies, expressed as mean normalisation expression (MNE) [Simon, 2003] using *ef1* as the reference gene to permit normalisation of the values, and calculated according to the following equation:

$$MNE_{target} = (E_{reference})^{Ct\ reference}/(E_{target})^{Ct\ target}.$$

Relative expression amounts were then expressed as the % Maximum MNE value ($= MNE_{target} \times 100/MNE_{maximum}$) calculated for each organ individually or with respect to testis MNE value.

Statistical Analysis

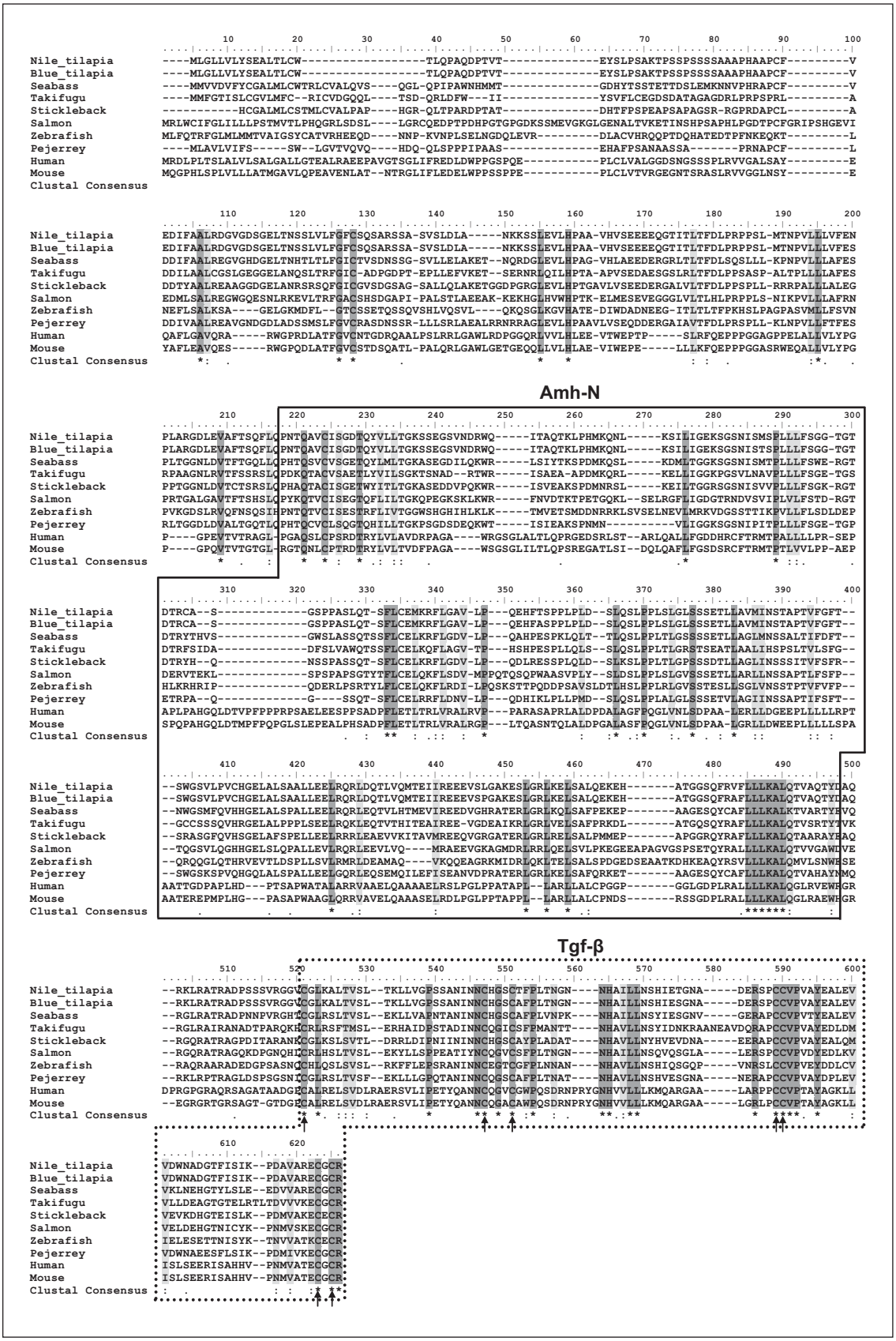
The data is presented as means ± standard error of the mean (SEM), and grouped in 3 different stages as 10–15 dpf, 17–19 dpf and 20–26 dpf. Significant differences in *amh* expression within a genotype XX or XY at the 3 dpf stages were analysed by ANOVA and mean differences were analysed by Tukey's post hoc test using XLSTAT software. Differences between the genotypes XX and XY for a particular dpf stage were analysed using Student t test. Differences were considered as statistically significant at $p \leq 0.05$. For the analysis using the 4 types of tissues, we performed a general linear modelling for the 4 independent factors analysed using Statistica 6.1 with %max MNE as dependent variable while tissue, genotype and dpf were explanatory independent variables.

Results

Characterisation of the Tilapia amh *Gene and Phylogenetic Analysis of the Protein*

We cloned the tilapia *amh* gene in order to verify if spliced forms existed and to assess the expression patterns of *amh* in the brain and gonad of tilapia during sex differentiation. The tilapia *amh* was cloned using both testis cDNA and genomic DNA. The open reading frame (ORF) of 1,545 bp in length encodes a predicted protein of 514-amino-acid residues (EF512167). The derived protein sequence contains a Tgf-β domain at the C-terminal part and an N-terminal Amh domain, both being characteristic of the Amh protein family (fig. 1). In the Amh-domain and before the beginning of the Tgf-β domain 2 typical protease cleavage sites (R-XX-R-XX-R) were identified from residues 404 until 410 (positions 503–509 in fig. 1 due to alignment) and at least one of them is necessary to produce the functional Amh [Rey et al., 2003]. The translated sequence shows that all of the 7 cysteine residues at the Tgf-β domain are in conserved positions as is typical for vertebrate Amhs. Furthermore, the positions of the 2 cysteine residues inside the Amh domain were also conserved with respect to all other teleost-derived sequences (positions 224 and 335 in fig. 1) but differ from

Fig. 1. Amino acid sequence alignment of Nile tilapia Amh protein and Amh of other teleosts, together with the AMH sequences of mouse and humans. Regions of amino acid similarity are shaded with identical amino acids indicated by asterisks and dark grey colour and identical residues based on average pairwise BLOSUM62 score in light grey. Conserved substitutions (same amino acid group properties) are indicated by colon (:), semi-conserved substitutions (similar amino acid structure) are indicated by a dot (.). The conserved cysteine-residues of the Tgf-β domain are indicated by arrows. The sequence framed with black lines corresponds to the predicted Amh-N domain while the dotted lines frame the predicted Tgf-β domain. The double protease cleavage site (R-XX-R-XX-R) corresponds in tilapia Amh to amino acid residues 404–410, although it appears, due to the alignment, at residues 503–509. Protein accession numbers of the sequences are given in Materials and Methods.


Amh-N
Tgf-β
Nile_tilapia
Blue_tilapia
Seabass
Takifugu
Stickleback
Salmon
Zebrafish
Pejerrey
Human
Mouse
Clustal Consensus

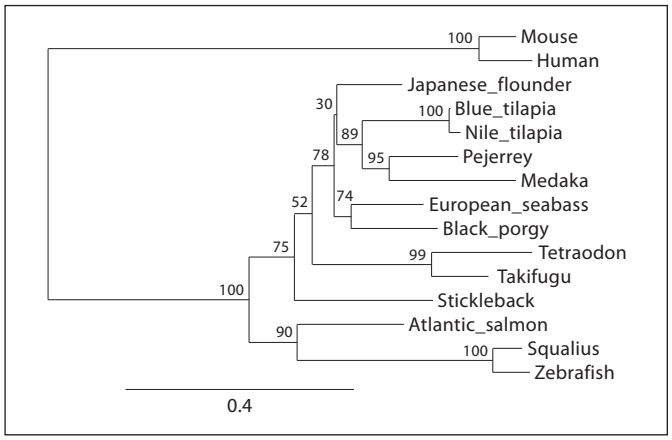


Fig. 2. Phylogenetic tree showing the relationship of Nile tilapia Amh and Amh of other species. Numbers indicate bootstrap values. Accession numbers of proteins used to establish the tree are given in Materials and Methods.

that of mammal Amh, where one of the conserved cysteine residues from the Amh domain is at another position (283) (fig. 1). Sequence homologies performed by BLASTN gave systematically hits with known vertebrate Amh sequences from NCBI GenBank followed by hits to the Bmp/Tgf-β sequences. This observation is clearly apparent with the phylogenetic analysis of Amh predicted protein sequences from teleosts (fig. 2), showing the evolutionary distance of the Nile tilapia sequence with that of mammals. The predicted Nile tilapia Amh sequence shared 98.6% overall identity with the blue tilapia ortholog, 62% with the sea bass Amh and 35.2% with the zebrafish Amh. The overall identity with mammal Amh is between 25 and 26% (table 1). The N-terminally located Amh-domain is not very conserved in other Perciformes since similarities ranged only from 62 to 64%. In contrast, the Tgf-β domain of the protein was more conserved, with 72% identity with the sea bass Amh and 38% identity with human and mouse AMH proteins.

As expected Nile tilapia Amh had the highest similarity to blue tilapia Amh, and they were phylogenetically most closely related to a clade formed by pejerrey and medaka Amh proteins and grouped in a larger clade containing the Japanese flounder Amh (fig. 2) probably brought about by the conserved similarity in the Tgf-β domain (table 1). Although, after the blue tilapia, we found the highest percentage of amino acid similarities between the Nile tilapia and sea bass Amh (table 1) when comparing the amino acids over the whole protein and the Amh-domain, the neighbour-joining analysis placed the sea bass in an evolutionarily distant clade containing the porgy Amh protein. This clade grouped with the larger clade containing the tilapia Amh, which branched separately to clades formed by the tetraodon, stickleback and zebrafish Amh proteins. All teleost Amhs were evolutionarily distant from that of mammals since they grouped in 1 main clade clearly separated from the mammalian AMH clade.

The phylogenetic closeness between Nile and blue tilapia is reflected in the high accordance of both *amh* genes showing 98% similarity also at the genomic DNA level. The Nile tilapia *amh* gene consists of 7 exons and 6 introns (fig. 3). Exons 4, 5, 6 and 7 encode the conserved Amh- and Tgf-β domains which are well conserved in teleosts. Medaka and tilapia genes were extremely similar in exon and intron sizes, with, for example, exon 7 differing in size by only 3 nucleotides between medaka and tilapias. All intron borders show the conserved signature GT//AG. Differentially spliced gene forms were not observed at any time in the testis DNA amplifications when using different primers. Recently, a partial Nile tilapia *amh* sequence (parts from exon 6 and intron 6; AM232733) was mapped on linkage group 23 [Shirak et al., 2006]. This 522-bp sequence fits with our *amh* sequence description, in which we have found 8 different bases in intron 6 due to polymorphism. Our sequence analysis data indicates clearly that the gene we have cloned is the Nile tilapia *amh* ortholog and it is nested clearly within the group of teleostei.

Expression of amh *mRNA in Adult Male and Female Organs*

The gonads have commonly been considered the only site of Amh production. In order to define which organs express the *amh* transcript in tilapia, real-time PCR quantification was performed in several adult organs from both males and females. Dimorphic expression of *amh* was seen in both gonads and brains. The highest *amh* MNE value (100%) was obtained in adult testis (mean = 80%) with much lower levels (mean = 34%) in ovaries (fig. 4a). *Amh* expression levels in male brains (mean = 24%) were slightly lower than in ovaries, while female brains expressed reduced amounts (mean = 8.7%). Male eyes had 5%, male spleens 6% and male head-kidney, kidney and intestine around 2%, while levels in females were all below 1%. *Amh* expression levels in pituitaries were low (~1.5%) and comparable between males and females. In the rest of the organs analysed, *amh* could be detected when using real-time PCR but MNE levels were only of 1% or below and thus considered negligible (fig. 4b).

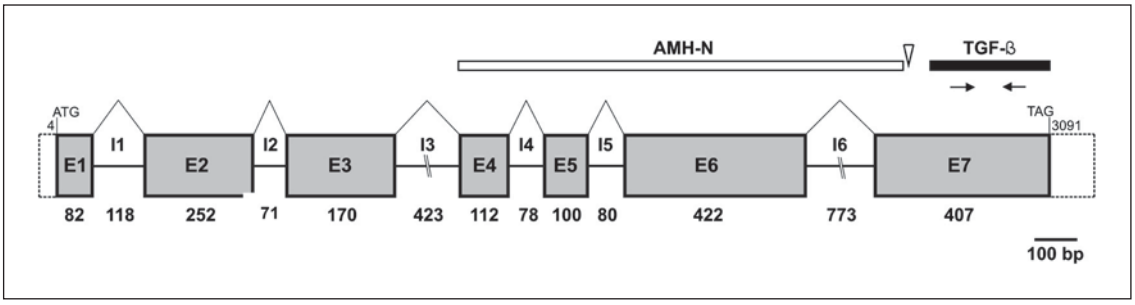


Fig. 3. Nile tilapia *amh* gene structure with the 7 exons in grey rectangles (E) and introns (I) shown as triangles. Sizes of both exons and introns are indicated below. The putative Amh-N domain is shown as a white bar, the Tgf-β domain as a black bar. The double protease cleavage site is indicated by a white arrowhead.

Table 1. Percentage of amino acid similarity between the Amh protein of Nile tilapia and Amh of various species (the similarity was calculated for the entire protein and for each domain)

Species	Order	Complete deduced amino acid sequence	% Amino acid similarity to Nile tilapia Amh protein		
			Entire protein	Amh domain	Tgf-β domain
Blue tilapia	Perciformes	514	98.64	98.31	97.84
Seabass	Perciformes	533	62.06	64.13	72.04
Pejerry	Atheriniformes	510	59.14	57.8	72.04
Japanese flounder	Pleuronectiformes	498	58.37	62.45	72.04
Black porgy	Perciformes	533	57.59	62.02	62.36
Stickleback	Gasterosteiformes	525	54.67	59.91	61.29
Medaka	Beloniformes	514	50.97	48.52	70.97
Takifugu	Tetraodontiformes	530	45.53	51.48	50.54
Salmon	Salmoniformes	571	45.53	47.26	62.36
Squalius	Cypriniformes	538	37.16	38.81	56.99
Zebrafish	Cypriniformes	549	35.21	35.86	54.84
Human	Primates	560	26.07	25.74	37.63
Mouse	Rodentia	554	24.71	24.05	37.63

Amh *Expression Profiles in Tilapia Heads during Sex Differentiation*

We underwent the present study to determine whether the developing brain of tilapia can also be a source of Amh. We analysed the expression levels of the *amh* gene in fry heads. Expression profiles were analysed at 3 major stages of sex differentiation by quantitative real-time PCR. The first stage (10–15 dpf) encompasses the period when the gonad is still undifferentiated and labile to sex reversal by external factors. The second stage which we sampled at 17–19 dpf corresponds to a period when sex can no longer be sex-reversed and is characterised by the first appearance of a sex difference, i.e. the germ cell number being higher in female gonads. The third period, 20–26 dpf, corresponds to an active ovarian mitosis, just before ovarian meiosis (~28 dpf) which is the stage when the ovary is considered morphologically differentiated [D'Cotta et al., 2001; Kobayashi and Nagahama, 2009].

The heads of XY males had astonishing high levels of *amh* expression at the very first stage of sex differentiation between 10 and 15 dpf (fig. 5a, b, 6a, b), considered the critical stage. In order to clearly establish that the *amh* expression detected in tilapia heads is restricted to the brain, we analysed separately the brains, gills and eyes from XX females and XY males at 10–14 dpf and compared the *amh* expression on whole heads (fig. 5a, b). *Amh* expression in tilapia male heads is primarily in the brain. If we consider that the brain corresponds to 100%, we

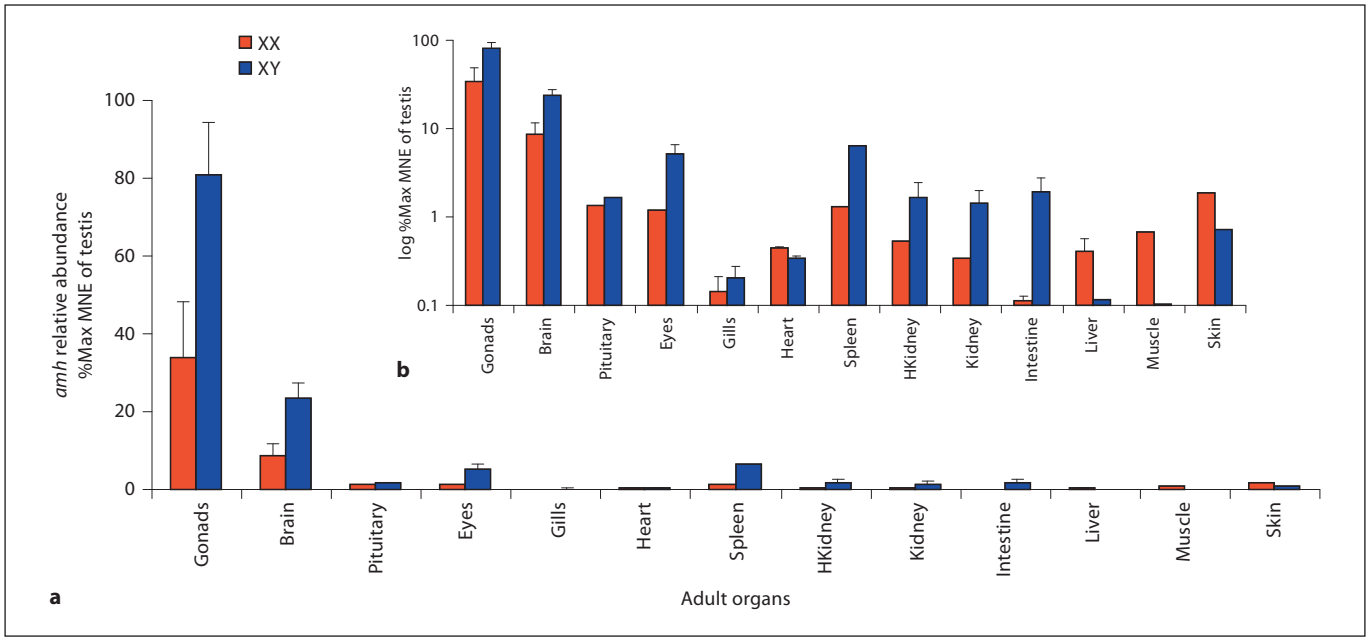


Fig. 4. Relative abundance of *amh* expression in organs of adult male and female tilapia quantified by real-time PCR. Males are shown in blue, females in red. **a** Values are represented as %Maximum Mean Normalisation Expression (%Max MNE) with regard to testis. **b** Log values of %Max MNE. For brain and gonads SEM was calculated for n = 3 biological samples, the remaining SEM values refer to n = 2.

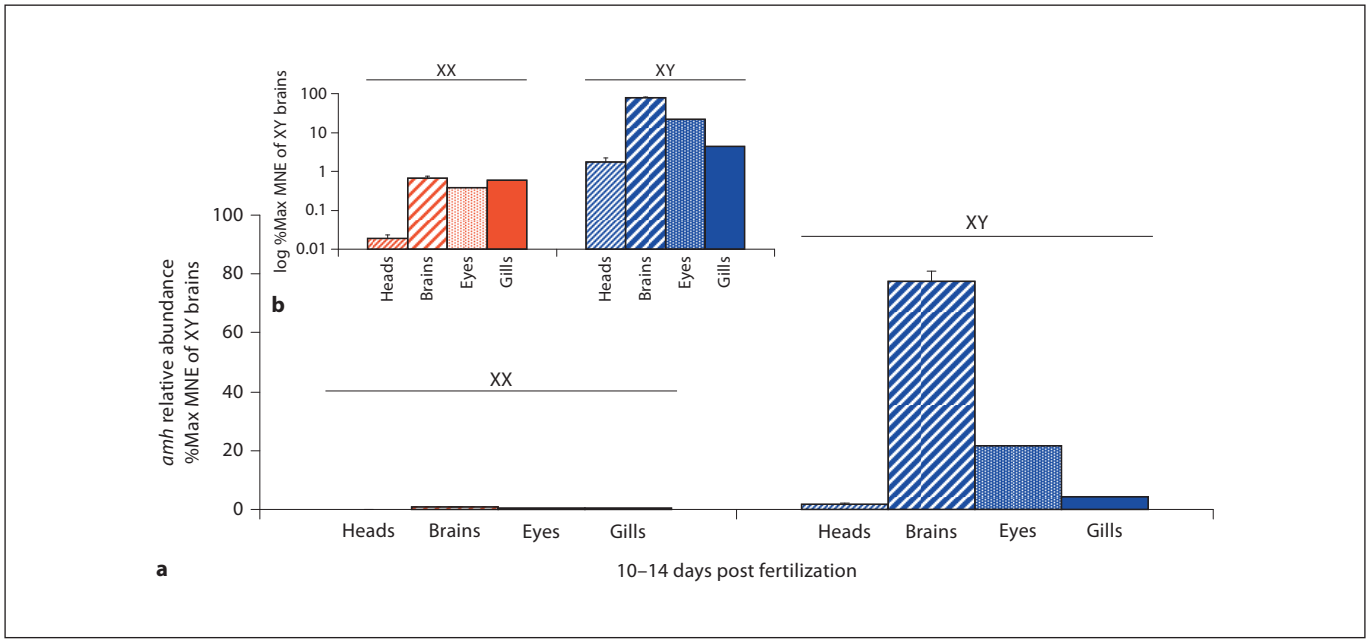


Fig. 5. Comparisons of *amh* gene expression found in tilapia XY male and XX female heads, brains, eyes and gills quantified by real-time PCR during the period of sex differentiation from 10 to 14 dpf. Male histograms are in blue, female histograms in red. **a** Values are represented as %Maximum MNE (%Max MNE) with respect to the maximum value found in XY brains. **b** Log values of %Max MNE levels. Values correspond to the mean values ± SEM (n = 2–6), except for eyes and gills (n = 1).

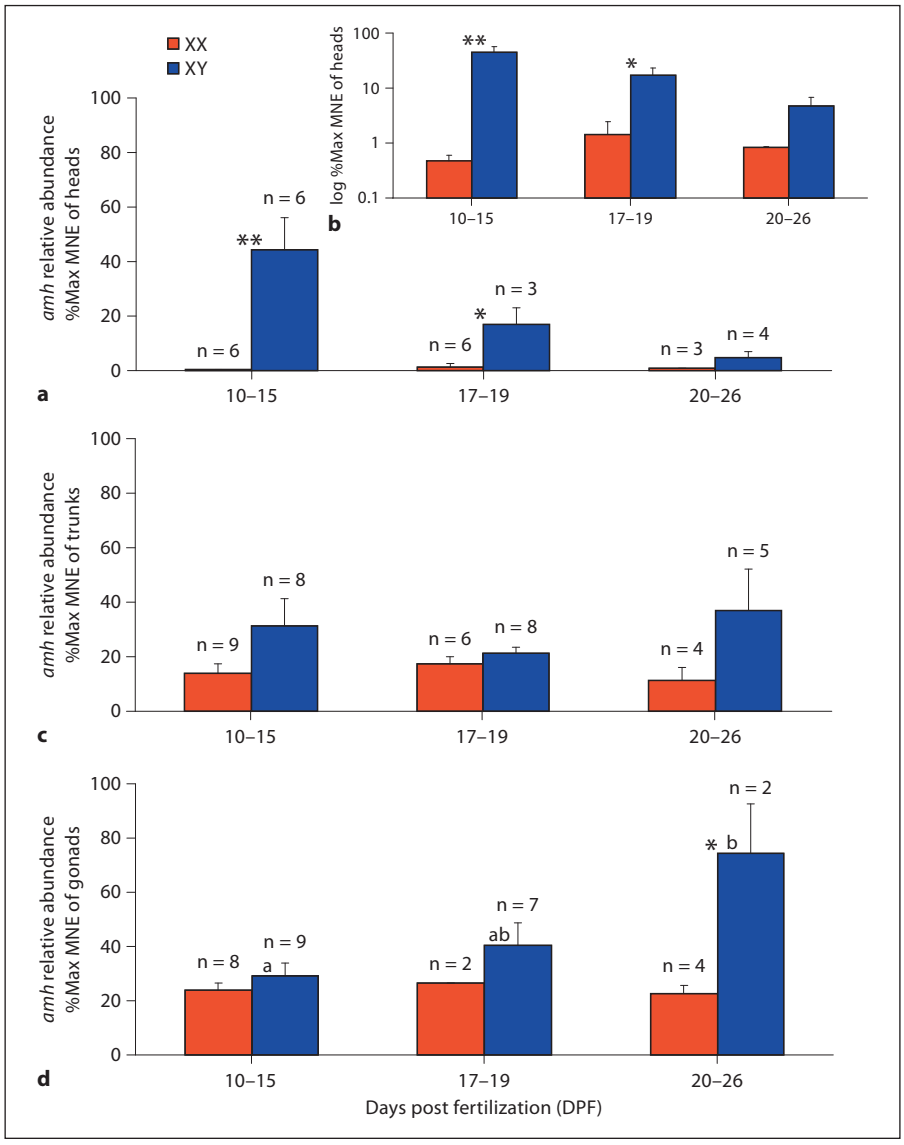


Fig. 6. Relative abundance of *amh* gene expression quantified by real-time PCR in tilapia XY males and XX females during the period of sex differentiation from 10 to 26 dpf. **a** Relative abundance of *amh* expression in heads given as %Maximum MNE (%Max MNE) with respect to the maximum value found in heads. **b** Log values of %Max MNE in heads. **c** Relative abundance of *amh* expression in trunks given as %Max MNE with respect to the maximum value found in trunks. **d** Relative abundance of *amh* expression in gonads given as %Max MNE with respect to the maximum value found in testes. Males are shown in blue, females in red. Values correspond to the mean values ± standard error of the means with the number of progenies (n) shown above each histogram. For each progeny at least 25 trunks were sampled and pooled. Asterisks denote: ** $p < 0.01$; * $p < 0.05$ found between XY males and XX females at a particular dpf. Different letters indicate significant differences $p < 0.05$ found between dpf for XY gonads.

found that the eyes contributed 27% and the gills around 5%. Hence the brain represents the majority of the head *amh* expression. Analysis on extra brain samples taken at 10–14 dpf confirmed the elevated *amh* expression detected in XY brains compared to XX females (fig. 5).

Throughout sex differentiation, XY males had consistently higher *amh* gene expression than XX females (fig. 6a, b) with levels being significantly different between sexes at 10–15 dpf ($p \leq 0.01$) and at 17–19 dpf ($p \leq 0.05$). After 15 dpf *amh* expressions steadily decreased in males to reach around the 20–26 dpf stage, comparable levels to those found in females. In females, *amh* amounts remained low throughout the sampling period.

Amh Expression Profiles in Tilapia Gonads and Trunks during Sex Differentiation

Pooled trunks containing gonads (fig. 6c) and pooled gonads (fig. 6d) were analysed at the 3 stages of tilapia sex differentiation. *Amh* was expressed in both XX female and XY male progenies from the early stage and throughout sex differentiation. Increased levels in males became apparent around 17–19 dpf and were clearly dimorphic in males between 20 and 26 dpf with overall higher levels in males. Male and female gonads presented statistical differences ($p \leq 0.05$) only at the 20–26 dpf stage (fig. 6d). *Amh* expression levels of XY gonads were different ($p \leq 0.05$) between the 10–15 and 20–26 dpf stages. Like we did for head *amh* analysis (fig. 6a, b), trunk and gonad *amh*

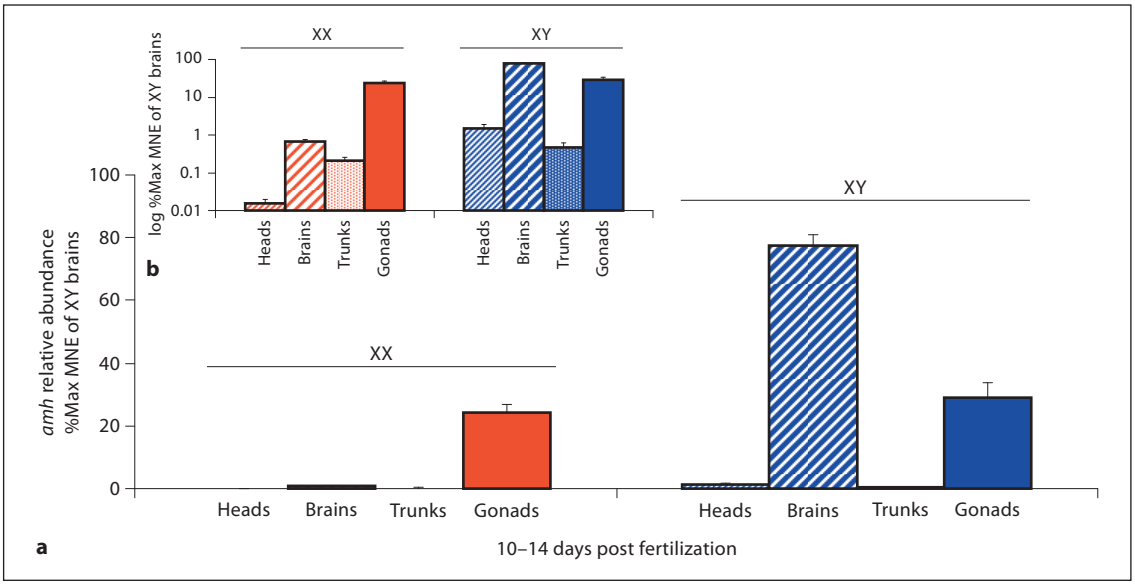


Fig. 7. Comparison of *amh* gene expression found in tilapia XY male and XX female heads, brains, trunks and gonads quantified by real-time PCR during the period of sex differentiation from 10 to 14 dpf. Male histograms are in blue, female histograms in red. **a** Values are represented as %maximum MNE (%Max MNE) with respect to the maximum value found in XY brains. **b** Log values of %Max MNE levels. Values correspond to the mean values ± SEM (n = 2–9).

MNE values were converted into percentage of the maximum value obtained in the trunk and gonads, respectively. This percentage permits calculation of relative amounts and a rapid visualisation without having a bias for the initial stages or for a particular sex.

Amh in sex-differentiating tilapia at 10–14 dpf is predominately expressed in XY brains (fig. 7). This is evident when relative expression levels were calculated for all organs as the percent maximum relative to brain values. The grouped analysis of *amh* expression in figure 7 shows in actual fact that male gonads have lower expression levels (2.7-fold lower) than brains during the early stages of sex differentiation. Levels of *amh* expression between male and female gonads were not significantly different at this stage. The linear model analysis on all tissues showed significant influences of tissue and genotype on % MNE. Among interaction effects, we found that tissue × dpf ($p = 0.03$), and tissue × genotype × dpf ($p = 0.02$) have significant effects on % MNE.

Discussion

The current study was performed to clone the complete *amh* gene in order to better characterise the gene and assess its involvement in the development of both male and female Nile tilapia. We were particularly interested in analysing whether *amh* is expressed elsewhere than in the gonads and if the developing brain of tilapia could be a source of Amh. This study shows that *amh* is expressed in adult brains of both male and female tilapias. We also put into evidence for the first time in a teleost that *amh* expression is dimorphic in tilapia brains with higher levels in males during the critical period of sex differentiation. *Amh* expression patterns were compared simultaneously between brains and gonads and assessed in XY males and XX females throughout the sex differentiation in tilapia.

To perform this study, we first cloned the entire *amh* gene in the Nile tilapia. Although the *amh* transcript has previously been quantified in the gonad by real-time PCR [Ijiri et al., 2008] and segregation studies have been performed [Shirak et al., 2006], the gene had not been cloned in tilapia nor differentially spliced forms searched. The Nile tilapia *amh* gene is formed by 7 exons like the *amh* gene of the European sea bass [Halm et al., 2007], medaka [Klüver et al., 2007] and zebrafish [Rodríguez-Marí et al., 2005] but differing from that of mammals [Rey et al., 2003] and birds [Oreal et al., 1998] which only have 5 exons. The 2 additional exons in teleosts are suggested to be due to intron insertions creating exon 2 and exon 7 [Halm et al., 2007]. The tilapia gene has intron and exon

sizes that resemble more those of the medaka *amh* gene than those of the sea bass gene. For instance, exons 4, 5 and 6 have identical sizes in tilapia and medaka. This is also true at the protein level, with similar amounts of residues. Nile tilapia Amh appears evolutionarily closer to medaka and pejerrey than to the group formed by sea bass and porgy Amhs despite these last being perciforms like tilapia. The predicted Amh protein of Nile tilapia has 514-amino-acid residues with a conserved Tgf-β domain containing the characteristic 7 cysteines. The Amh domain was much less conserved. We did not find alternatively spliced isoforms such as those described in the European sea bass, in which 2 additional spliced forms were expressed in relatively comparable levels to that of the unspliced form [Halm et al., 2007].

We analysed the tissue distribution of *amh* expression in adult tilapia and found that *amh* is preferentially expressed in testis but also in other organs, notably in the brain. *Amh* expression in male brains is 4-fold lower than in testis, this level being only slightly lower than in ovaries. Pituitaries had low levels of *amh* expression and were not different between males and females. Surprisingly we found that adult males had *amh* expression in both eyes and spleen (5 and 6%). *Amh* was also expressed in other organs such as intestine, liver, head-kidney and kidney, but levels were low to negligible (<1%). Since the pioneering finding of the first fish *amh* gene in the Japanese eel [Miura et al., 2002], an *amh* gene has been identified in a number of teleost fish despite them lacking müllerian ducts. In the majority of these teleosts, tissue distribution of *amh* expression was restricted to the gonads analyzed either in larvae or in adults (Japanese flounder [Yoshinaga et al., 2004]; zebrafish [von Hofsten et al., 2005]; medaka [Klüver et al., 2007], and pejerrey [Fernandino et al. 2008]). However, these studies might have overseen brain *amh* mostly due to the use of RT-PCR which is much less sensitive than real-time PCR applied in the current study. Indeed, using real-time PCR a recent work demonstrated *amh* expression in the brain of adult zebrafish together with expression in testis, muscle, eye, ovary, skin, kidney, liver and heart [Wang and Orban, 2007]. *Amh* expression was detected in the brain and pituitary of juvenile and adult European sea bass as well as in the testes, ovaries and heart when using southern blots with RT-PCR products [Halm et al., 2007].

In the current study, *amh* has been shown to have a sexual dimorphic expression with elevated levels in the developing brains of XY male Nile tilapias. *Amh* sex differences in the brain occur at very early stages, already being evident at 10 dpf and lasting till ~20 dpf. The 10–15-dpf period when brain *amh* in males was at its highest corresponds to the period (5–6 days post hatching dph = 10–11 dpf at 26°C) of the first sex-dimorphic expression of *cyp19a1a* and *foxl2* in the gonads of XX female fry/larvae, and *dmrt1* in male fry of the Nile tilapia [Ijiri et al., 2008]. The gonads at this stage have been considered still undifferentiated in particular from a histological point of view but also because they are still bipotential. They can be manipulated by external factors such as hormone treatments or temperature to functionally inverse the sex of the gonad, if treatments are applied from 10 dpf onwards [Baroiller et al., 2009]. We consider this stage the actual critical period of sex differentiation in tilapia, being the period when the decision towards the development of ovaries or testes is being made, and it has been established from 9 to 15 dpf by our group [Baroiller et al., 2009] and that of Nagahama [Ijiri et al., 2008]. During this stage the gonads in both sexes are mainly composed of supporting cells with few primordial germ cells (PGC) [D'Cotta et al., 2001] with an increase in the number of PGC in XX females starting around 13–14 dpf (9–10 dph) [Kobayashi and Nagahama, 2009]. In comparison to the early sexual dimorphism found for brain *amh* expression, the gonads had similar *amh* expression in both XY males and XX females during the 10–15-dpf period. Following this period *amh* in the gonads was gradually up-regulated in XY males to become dimorphic only after 17 dpf. This timing is consistent with the results of Ijiri et al. [2008] who found in testis an up-regulation of *amh* at 19 dpf (15 dph) when studying *amh* expression together with other genes implicated in the sex-differentiating cascade.

In developing tilapia, we analysed the larvae/fry head as representative of the brain. In order to define if *amh* expression may in fact exist elsewhere than in the brain, we dissected part of the heads to analyse the expression pattern separately in the brain, gills and eyes at 14 dpf. The brain is the principal organ expressing *amh* in the head, and this was corroborated by additional analyses done on brains of other progenies. But interestingly, we also found *amh* expressed in developing eyes at a 4-fold lower level than in brains. The comparison of *amh* expression in male and female brains confirmed the large sex dimorphism of *amh* at 14 dpf observed in the heads. *Amh* was expressed in male larvae brains at 2.7-fold higher levels than in testis. The period when *amh* expression is elevated in male brains is relatively short with levels later decreasing substantially to low levels. This may be one of the reasons why only a few studies performed in some vertebrate classes have found *amh* expressed in the brain. In mice, Wang et al. [2005] detected *Amh* expres-

sion in the brain only after having isolated motor neurons by laser-capture. Taken together, the finding of *amh* in the brain of tilapia, sea bass and zebrafish emphasises the fact that *amh* may play a role in the regulation of the brain-gonadal axis in these fish, but the mechanism of Amh action and its targets are unknown.

The possible role of Amh during teleost development, and more particularly the likely involvement of Amh in the brain has still to be deciphered. Because teleosts lack müllerian ducts, Amh action is other than leading to their regression. In differentiating testis *amh* is expressed in supporting pre-Sertoli cells in the Japanese flounder [Yoshinaga et al., 2004], Japanese eel [Miura et al., 2002], zebrafish [Rodríguez-Marí et al., 2005], pejerrey [Fernandino et al., 2008] and medaka [Klüver et al., 2007]. We presume that expression in tilapia is also in these supporting cells. AMHR2 is the first target of AMH signalling which has considerably been studied in mammals [Teixeira et al., 1999; Josso et al., 2001]. In fish, *amhr2* has, as yet, only been identified in medaka which in contrast to other species had no sex-dimorphic expression for *amh* or *amhr2* in the gonads during sex differentiation [Klüver et al., 2007]. In medaka, Amh signalling has been shown to be involved in germ cell proliferation during early gonad development in both sexes. Mutation of *amhr2* caused a dysregulation of germ cell proliferation [Morinaga et al., 2007] and loss-of-function experiments showed that embryos with deficient *amh* and *amhr2* had lower numbers of germ cells in both XY and XX gonads [Shiraishi et al., 2008]. It is plausible that Amh in tilapia gonads may be indirectly involved in germ cell proliferation, perhaps regulating in males the proliferation of germ cells, but this has still to be explored.

AMH in mammals persists in Sertoli cells till the onset of puberty and is involved in the negative regulation of spermatogenesis [Josso and Di Clemente, 2003]. AMH is also detected after birth in the granulosa cells in ovaries regulating follicle maturation [Ueno et al., 1989; Durlinger et al., 2002]. In adult fish, *amh* expression also persists in Sertoli cells of testis and was shown in the Japanese eel to suppress spermatogenesis, with expression disappearing upon spermatogonial maturation [Miura et al., 2002]. Differing from eel, adult zebrafish had *amh* expression in granulosa cells together with *cyp19a1a* suggesting that Amh might be involved in follicle growth but not maturation like in mammals [Rodríguez-Marí et al., 2005].

One of the hypotheses of Amh function in teleost gonads is that it could be an anti-aromatase factor as reported in mammals. In foetal ovaries aromatase activity was inhibited in vitro, blocking estradiol (E2) synthesis and causing a change in steroid production, with the secretion of testosterone instead of E2 [Vigier et al., 1989]. AMH inhibitory effects were shown to be through the suppression of the aromatase *CYP19* gene [Di Clemente et al., 1992]. In cultured human granulosa-lutein cells, AMH inhibited the FSH-stimulated E2 synthesis, diminishing both the gene expression and protein levels of CYP19 [Grossman et al., 2008]. In teleosts, the possible interactions between Amh and Cyp19 have also been searched in gonads during sex differentiation. This is particularly noteworthy because endogenous estrogens have long been known to function in teleosts as natural inducers of ovarian differentiation [Guiguen et al., 1999; D'Cotta et al., 2001; Devlin and Nagahama, 2002]. In tilapia, female-to-male reversal occurs in XX individuals if the production of E2 is disrupted when using an inhibitor of aromatase activity during sex differentiation [Guiguen et al., 1999; Kwon et al., 2000]. Reciprocal expression between *amh* and the gonad aromatase gene *cyp19a1a* have been found in differentiating gonads of rainbow trout [Vizziano et al, 2008], in the TSD pejerrey [Fernandino et al., 2008], Japanese flounder [Kitano et al., 2007] and tilapia [Ijiri et al., 2008; Poonlaphdecha et al., unpubl. data]. These studies show that *amh* in the gonads is modulated by estrogen levels as well as by temperature, but the earlier expression of *cyp19a1a* in differentiating tilapia XX females [Ijiri et al., 2008; Poonlaphdecha et al., unpubl. data] suggests that Amh in tilapia is not an inhibitor of *cyp19a1a*.

There is a long-standing view that gonadal steroid hormones induce most of the sexual differentiation of the brain in mammals and birds. Testosterone is secreted in foetal testes and in the brain and is metabolised into E2 by aromatase or to dihydrotestosterone, to induce the sexual dimorphic organization of the brain [Davies and Wilkinson, 2006]. Sex differences have been found in the brain prior to gonad differentiation and hormone secretion in mice [Dewing et al., 2003] and birds [Scholz et al., 2006], challenging the old dogma that male and female brain differences only occurred after exposure to gonadal hormones. Studies indicate that some morphological sex differences of the brain are induced by genetic influences [Arnold et al., 2004]. We have shown in the present study that *amh* expression differs in brains of genetic XY male and XX female tilapias from 10 to 15 dpf, when the first dimorphic expression of *cyp19a1a, foxl2* and *dmrt1* also occurred in the undifferentiated gonads. At this period E2 production in the gonad most probably is still low, judged by the low number of aromatase-producing cells

[Devlin and Nagahama, 2002] and the relatively low *cyp19a1a* transcript amounts, in spite of the sex dimorphism of some gonadal genes [Ijiri et al., 2008]. In tilapia, the earliest measurements of E2 levels were performed on pooled gonads from 18 to 26 dpf, showing that XX females had 5-fold higher levels of E2 than XY males [Baroiller and D'Cotta, 2001].

Estrogen is synthesised in the brain of teleosts [Callard et al., 2001]. Aromatase activity is astonishingly elevated in teleost brain, particularly in the forebrain (telencephalon and diencephalon) and the pituitary, and may stem from the continuous neurogenesis of fish [Callard et al., 2001; Diotel et al., 2010]. We have shown in previous studies that tilapia brains had already elevated aromatase activity at 18 dpf when gonads are still not differentiated [D'Cotta et al., 2001]. At this stage XX female brains had higher aromatase activity than XY males, and temperature masculinisation caused a suppression of aromatase activity of XX females but also of XY males [D'Cotta et al., 2001]. The brain aromatase form *cyp19a1b* appears from 4 dpf onwards in tilapia brains, but surprisingly no sex dimorphism has been observed during the gonad sex differentiating period by other authors [Kwon et al., 2001; Chang et al., 2005] or by us, although we did find sex dimorphism for *foxl2* [Poonlaphdecha et al., unpubl. observations]. In tilapia, *foxl2* has been shown to activate the *cyp19a1a* gene in vitro [Wang et al., 2007]. Work on *foxl2* and *cyp19a1b* suggests that they may regulate sex differentiation in the brain since E2 caused an up-regulation of these genes while fadrozole caused a down-regulation in the brain of Southern catfish [Liu et al., 2007]. Hence, the brain during sex differentiation has all the potential for local E2 production. It is plausible that Amh may have an anti-aromatase effect in brain of male tilapia, but in this case Amh action would be on the aromatase activity rather than on *cyp19a1b* transcription since no sex differences have been shown for this gene. Elevated *amh* levels in the developing brain of XY males may be to ensure masculinisation of the brain. Amh could perhaps be involved in organizing the male brain so that it can regulate the E2 production, not only in the brain but perhaps it also has implications at the gonadal level.

Recent studies in mammals suggest that AMH should now be considered as a new hormone in the brain [Wang et al., 2005; Lebeurrier et al., 2008]. Expression of *Amh* and *Amhr2* has been evidenced in mouse brain, and this AMH-dependent signalling pathway controls the survival of motor neurons [Wang et al., 2005]. *Amh* expression was also revealed in rat brain and shown to be a regulator of neuroserpin expression functioning as a neuroprotective factor [Lebeurrier et al., 2008]. In addition, *Amhr2* was expressed in the brain of developing mice, and in male $Amh^{-/-}$ and $Amhr2^{-/-}$ mice some feminisation of the spinal motor neurons occurred with repercussions in their behaviour [Wang et al., 2009]. These studies together with work on tilapia suggest that *amh* may be a new brain hormone, and raises the possibility of its participation in the sex differentiation of the brain during development. Earlier work on a murine gonadotrope-derived cell line suggested that AMH could modulate the hypothalamic-pituitary-gonadal axis [Bédécarrats et al., 2003] because the addition of AMH to the cell line induced a rapid up-regulation of *Fshb* expression. AMH in synergy with the *Fshb* promoter enhanced the stimulatory effect of gonadotropin-releasing hormone (GnRH) on the luteinizing hormone β (LHβ) gene promoter [Bédécarrats et al., 2003].

Early sexual dimorphism of tilapia brain has been seen for the seabream GnRH in the preoptic area-hypothalamus (POA-H) with higher immunolocalisation in XY from 9 to 14 dpf, and later in XY and XX pituitaries [Swapna et al., 2008]. This sbGnRH is considered the main form involved in gonadotropin release with the GnRH-POA-H system being important for sexual maturation, sexual behaviour and for synchronising gonadal cycles [Senthilkumaran et al., 1999]. This increase in male POA-H neurons coincides with the elevated *amh* expression of males seen in the present study. Both *fshb* and *lhb* gene expression and protein detection have been evidenced in the brain and pituitary of tilapia already at 14 dpf, suggesting that they may also regulate gonad sex differentiation apart from other reproductive actions as well as functioning as neuromodulators of reproductive behaviour [Parhar et al., 2003]. Additional evidence that sex differences already exist in the brain at 14 dpf has been shown for tryptophan hydroxylase (Tph) with higher expression in males than in females [Sudhakumari et al., 2010]. Tph is an enzyme involved in serotonin (5HT) synthesis functioning as a neurotransmitter, but it appears to also stimulate GnRH release and regulate that of gonadotropins [Senthilkumaran et al., 2001]. A relationship with aromatase activity was suggested when Tph was blocked, reducing 5HT levels in brains during tilapia ontogenesis which caused an increase in female proportions [Tsai et al., 2000]. The possible interaction of Amh with these different actors in tilapia brain has still to be proven.

In conclusion, we have found that XY males have elevated *amh* expression in the brain at the onset of gonad differentiation and before *amh* expression in the gonads, suggesting that brain sex differentiation is initiated si-

multaneously and perhaps even before gonad steroid production. We suggest that elevated *amh* expression levels in the developing brain of males may ensure a masculinisation of the brain. This could be through an anti-aromatase role, regulating brain E2 levels. Amh appears to possess multiple functions during the sex differentiation of tilapia. The high *amh* expression of male brains could be used as a precocious sexing procedure for tilapias. An interesting question is what exactly does dimorphic expression of *amh* in the brain mean? Are there differences in cell number or differences in expression levels for each cell between males and females? The study of the Amh protein as well as its receptor and assessing in what regions of the brain they are observed would enlighten the possible role played by Amh in tilapia brain.

Acknowledgements

This work was supported partially by the ANR-Genanimal Fishsex project (ANR-O6-GANI-012) and by the CIRAD. S.P. was funded by the CIRAD, S.-H.H. was funded by the Institut Français de Taipei and the National Sun Yat-Sen University.

References

Arango NA, Lovell-Badge R, Behringer RR: Targeted mutagenesis of the endogenous mouse *Mis* gene promoter: in vivo definition of genetic pathways of vertebrate sexual development. Cell 99:409–419 (1999).

Arnold AP, Xu J, Grisham W, Chen X, Kim YH, Itoh Y: Sex chromosomes and brain sexual differentiation. Endocrinology 145:1057–1062 (2004).

Baroiller JF, D'Cotta H: Environment and sex determination in farmed fish. Comp Biochem Physiol C Toxicol Pharmacol 130:399–409 (2001).

Baroiller JF, Jalabert B: Contribution of research in reproductive physiology to the culture of tilapias. Aquat Living Resour 2:105–116 (1989).

Baroiller JF, D'Cotta H, Bezault E, Wessels S, Hoerstgen-Schwark G: Tilapia sex determination: Where temperature and genetics meet. Comp Biochem Physiol A Mol Integr Physiol 153:30–38 (2009).

Bédécarrats GY, O'Neill FH, Norwitz ER, Kaiser UB, Teixeira J: Regulation of gonadotropin gene expression by müllerian inhibiting substance. Proc Natl Acad Sci USA 100:9348–9353 (2003).

Callard GV, Tchoudakova AV, Kishida M, Wood E: Differential tissue distribution, developmental programming, estrogen regulation and promoter characteristics of *cyp19* genes in teleost fish. J Steroid Biochem Mol Biol 79:305–314 (2001).

Chang X, Kobayashi T, Senthilkumaran B, Kobayashi-Kajura H, Sudhakumari CC, Nagahama Y: Two types of aromatase with different encoding genes, tissue distribution and developmental expression in Nile tilapia *(Oreochromis niloticus)*. Gen Comp Endocrinol 141:101–115 (2005).

Chevenet F, Brun C, Banuls AL, Jacq B, Christen R: TreeDyn: towards dynamic graphics and annotations for analyses of trees. BMC Bioinformatics 7:439 (2006).

Davies W, Wilkinson LS: It is not all hormones: alternative explanations for sexual differentiation of the brain. Brain Res 1126:36–45 (2006).

D'Cotta H, Fostier A, Guiguen Y, Govoroun M, Baroiller JF: Aromatase plays a key role during normal and temperature-induced sex differentiation of tilapia *Oreochromis niloticus*. Mol Reprod Dev 59:265–276 (2001).

Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, et al: Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Res 36:465–469 (2008).

De Santa Barbara P, Bonneaud N, Boizet B, Desclozeaux M, Moniot B, et al: Direct interaction of SRY-related protein SOX9 and steroidogenic factor 1 regulates transcription of the human anti-müllerian hormone gene. Mol Cell Biol 18:6653–6665 (1998).

Devlin RH, Nagahama Y: Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 208:191–364 (2002).

Dewing P, Shi T, Horvath S, Vilain E: Sexually dimorphic gene expression in mouse brain precedes gonadal differentiation. Brain Res Mol Brain Res 118:82–90 (2003).

Di Clemente N, Ghaffari S, Pepinsky RB, Pieau C, Josso N, et al: A quantitative and interspecific test for biological activity of anti-müllerian hormone: the fetal ovary aromatase assay. Development 114:721–727 (1992).

Diotel N, Page YL, Mouriec K, Tong SK, Pellegrini E, et al: Aromatase in the brain of teleost fish: expression, regulation and putative functions. Front Neuroendocrinol 31:172–192 (2010).

Durlinger AL, Visser JA, Themmen AP: Regulation of ovarian function: the role of anti-müllerian hormone. Reproduction 124:601–609 (2002).

Edgar RC: MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797 (2004).

Fernandino JI, Hattori RS, Kimura H, Strüssmann CA, Somoza GM: Expression profile and estrogenic regulation of anti-müllerian hormone during gonadal development in pejerrey *Odontesthes bonariensis*, a teleost fish with strong temperature-dependent sex determination. Dev Dyn 237:3192–3199 (2008).

Grossman MP, Nakajima ST, Fallat ME, Siow Y: Müllerian-inhibiting substance inhibits cytochrome P450 aromatase activity in human granulosa lutein cell culture. Fertil Steril 89:1364–1370 (2008).

Guiguen Y, Baroiller JF, Ricordel MJ, Iseki K, Mcmeel OM, et al: Involvement of estrogens in the process of sex differentiation in two fish species: the rainbow trout *(Oncorhynchus mykiss)* and a tilapia *(Oreochromis niloticus)*. Mol Reprod Dev 54:154–162 (1999).

Halm S, Rocha A, Miura T, Prat F, Zanuy S: Anti-müllerian hormone (*AMH*/AMH) in the European sea bass: its gene structure, regulatory elements, and the expression of alternatively-spliced isoforms. Gene 388:148–158 (2007).

Ijiri S, Kaneko H, Kobayashi T, Wang DS, Sakai F, et al: Sexual dimorphic expression of genes in gonads during early differentiation of a teleost fish, the Nile tilapia *Oreochromis niloticus*. Biol Reprod 78:333–341 (2008).

Josso N, Di Clemente N: Transduction pathway of anti-müllerian hormone, a sex-specific member of the TGF-beta family. Trends Endocrinol Metab 14:91–97 (2003).

Josso N, Di Clemente N, Gouédard L: Anti-müllerian hormone and its receptors. Mol Cell Endocrinol 179:25–32 (2001).

Kitano T, Yoshinaga N, Shiraishi E, Koyanagi T, Abe S: Tamoxifen induces masculinization of genetic females and regulates P450 aromatase and müllerian inhibiting substance mRNA expression in Japanese flounder *(Paralichthys olivaceus)*. Mol Reprod Dev 74:1171–1177 (2007).

Klüver N, Pfennig F, Pala I, Storch K, Schlieder M, et al: Differential expression of anti-müllerian hormone *(amh)* and anti-müllerian hormone receptor type II *(amhrII)* in the teleost medaka. Dev Dyn 236:271–281 (2007).

Kobayashi T, Nagahama Y: Molecular aspects of gonadal differentiation in a teleost fish, the Nile tilapia. Sex Dev 3:108–117 (2009).

Kwon JY, Haghpanah V, Kogson-Hurtado LM, McAndrew BJ, Penman DJ: Masculinization of genetic female Nile tilapia *(Oreochromis niloticus)* by dietary administration of an aromatase inhibitor during sexual differentiation. J Exp Zool 287:46–53 (2000).

Kwon JY, McAndrew BJ, Penman DJ: Cloning of brain aromatase gene and expression of brain and ovarian aromatase genes during sexual differentiation in genetic male and female Nile tilapia *Oreochromis niloticus*. Mol Reprod Dev 59:359–370 (2001).

Lebeurrier N, Launay S, Macrez R, Maubert E, Legros H, et al: Anti-müllerian-hormone-dependent regulation of the brain serine-protease inhibitor neuroserpin. J Cell Sci 121:3357–3365 (2008).

Liu Z, Wu F, Jiao B, Zhang X, Hu C, et al: Molecular cloning of doublesex and mab-3-related transcription factor 1, forkhead transcription factor gene 2, and two types of cytochrome P450 aromatase in Southern catfish and their possible roles in sex differentiation. J Endocrinol 194:223–241 (2007).

Miura T, Miura C, Konda Y, Yamauchi K: Spermatogenesis-preventing substance in Japanese eel. Development 129:2689–2697 (2002).

Morinaga C, Saito D, Nakamura S, Sasaki T, Asakawa S, et al: The hotei mutation of medaka in the anti-mullerian hormone receptor causes the dysregulation of germ cell and sexual development. Proc Natl Acad Sci USA 104:9691–9696 (2007).

Morrish BC, Sinclair AH: Vertebrate sex determination: many means to an end. Reproduction 124:447–457 (2002).

Munsterberg A, Lovell-Badge R: Expression of the mouse anti-müllerian hormone gene suggests a role in both male and female sexual differentiation. Development 113:613–624 (1991).

Oreal E, Pieau C, Mattei MG, Josso N, Picard JY, et al: Early expression of *AMH* in chicken embryonic gonads precedes testicular *SOX9* expression. Dev Dyn 212:522–532 (1998).

Parhar IS, Soga T, Ogawa S, Sakuma Y: FSH and LH-beta subunits in the preoptic nucleus: ontogenic expression in teleost. Gen Comp Endocrinol 132:369–378 (2003).

Rey R, Lukas-Croisier C, Lasala C, Bedecarrás P: *AMH/MIS*: what we know already about the gene, the protein and its regulation. Mol Cell Endocrinol 211:21–31 (2003).

Rodríguez-Marí A, Yan YL, Bremiller RA, Wilson C, Cañestro C, Postlethwait JH: Characterization and expression pattern of zebrafish anti-müllerian hormone *(amh)* relative to *sox9a, sox9b,* and *cyp19a1a*, during gonad development. Gene Expr Patterns 5:655–667 (2005).

Rouiller-Fabre V, Carmona S, Merhi RA, Cate R, Habert R, Vigier B: Effect of anti-müllerian hormone on Sertoli and Leydig cell functions in fetal and immature rats. Endocrinology 139:1213–1220 (1998).

Saitou N, Nei M: The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425 (1987).

Scholz B, Kultima K, Mattsson A, Axelsson J, Brunström B, et al: Sex-dependent gene expression in early brain development of chicken embryos. BMC Neurosci 7:12 (2006).

Senthilkumaran B, Okuzawa K, Gen K, Ookura T, Kagawa H: Distribution and seasonal variations in levels of three native GnRHs in the brain and pituitary of perciform fish. J Neuroendocrinol 11:181–186 (1999).

Senthilkumaran B, Okuzawa K, Gen K, Kagawa H: Effects of serotonin, GABA and neuropeptide Y on seabream GnRH hormone release in vitro from preoptic-anterior hypothalamus and pituitary of red seabream, *Pagrus major*. J Neuroendocrinol 13:395–400 (2001).

Shiraishi E, Yoshinaga N, Miura T, Yokoi H, Wakamatsu Y, et al: Müllerian inhibiting substance is required for germ cell proliferation during early gonadal differentiation in medaka *(Oryzias latipes)*. Endocrinology 149:1813–1819 (2008).

Shirak A, Seroussi E, Cnaani A, Howe AE, Domokhovsky R, et al: *Amh* and *dmrta2* genes map to tilapia (*Oreochromis* spp.) linkage group 23 within quantitative trait locus regions for sex determination. Genetics 174: 1573–1581 (2006).

Shoemaker CM, Crews D: Analyzing the coordinated gene network underlying temperature-dependent sex determination in reptiles. Semin Cell Dev Biol 20:293–303 (2009).

Shoemaker C, Ramsey M, Queen J, Crews D: Expression of *Sox9, Mis*, and *Dmrt1* in the gonad of a species with temperature-dependent sex determination. Dev Dyn 236:1055–1063 (2007).

Simon P: Q-Gene: processing quantitative real-time RT-PCR data. Bioinformatics 19:1439–1440 (2003).

Smith CA, Sinclair AH: Sex determination: insights from the chicken. BioEssays 26:120–132 (2004).

Smith CA, Smith MJ, Sinclair AH: Gene expression during gonadogenesis in the chicken embryo. Gene 234:395–402 (1999).

Sudhakumari CC, Senthilkumaran B, Raghuveer K, Wang DS, Kobayashi T, et al: Dimorphic expression of tryptophan hydroxylase in the brain of XX and XY Nile tilapia during early development. Gen Comp Endocrinol 166:320–329 (2010).

Swapna I, Sudhakumari CC, Sakai F, Sreenivasulu G, Kobayashi T, et al: Seabream GnRH immunoreactivity in brain and pituitary of XX and XY Nile tilapia, *Oreochromis niloticus* during early development. J Exp Zool A, Ecol Genet Physiol 309:419–426 (2008).

Teixeira J, Kehas DJ, Antun R, Donahoe PK: Transcriptional regulation of the rat müllerian inhibiting substance type II receptor in rodent Leydig cells. Proc Natl Acad Sci USA 96:13831–13838 (1999).

Tremblay JJ, Viger RS: Transcription factor GATA-4 enhances müllerian inhibiting substance gene transcription through a direct interaction with the nuclear receptor SF-1. Mol Endocrinol 13:1388–1401 (1999).

Tsai CL, Wang LH, Chang CF, Kao CC: Effects of gonadal steroids on brain serotonergic and aromatase activity during the critical period of sexual differentiation in tilapia, *Oreochromis mossambicus*. J Neuroendocrinol 12: 894–898 (2000).

Ueno S, Takahashi M, Manganaro TF, Ragin RC, Donahoe PK: Cellular localization of müllerian inhibiting substance in the developing rat ovary. Endocrinology 124:1000–1006 (1989).

Vigier B, Forest MG, Eychenne B, Bézard J, Garrigou O, et al: Anti-müllerian hormone produces endocrine sex reversal of fetal ovaries. Proc Natl Acad Sci USA 86:3684–3688 (1989).

Vizziano D, Baron D, Randuineau G, Mahè S, Cauty C, Guiguen Y: Rainbow trout gonadal masculinization induced by inhibition of estrogen synthesis is more physiological than masculinization induced by androgen supplementation. Biol Reprod 78:939–946 (2008).

von Hofsten J, Larsson A, Olsson PE: Novel steroidogenic factor-1 homolog *(ff1d)* is coexpressed with anti-müllerian hormone *(AMH)* in zebrafish. Dev Dyn 233:595–604 (2005).

Wang DS, Kobayashi T, Zhou LY, Paul-Prasanth B, Ijiri S, et al: Foxl2 up-regulates aromatase gene transcription in a female-specific manner by binding to the promoter as well as interacting with ad4 binding protein/steroidogenic factor 1. Mol Endocrinol 21:712–725 (2007).

Wang PY, Koishi K, McGeachie AB, Kimber M, MacLaughlin DT, et al: Müllerian inhibiting substance acts as a motor neuron survival factor in vitro. Proc Natl Acad Sci USA 102: 16421–16425 (2005).

Wang PY, Protheroe A, Clarkson AN, Imhoff F, Koishi K, McLennan IS: Müllerian inhibiting substance contributes to sex-linked biases in the brain and behaviour. Proc Natl Acad Sci USA 106:7203–7208 (2009).

Wang XG, Orban L: Anti-Müllerian hormone and 11 beta-hydroxylase show reciprocal expression to that of aromatase in the transforming gonad of zebrafish males. Dev Dyn 236:1329–1338 (2007).

Western PS, Harry JL, Graves JA, Sinclair AH: Temperature dependent sex determination: upregulation of *SOX9* expression after commitment to male development. Dev Dyn 214: 171–177 (1999).

Wrobel KH: The genus *Acipenser* as a model for vertebrate urogenital development: the müllerian duct. Anat Embryol (Berl) 206:255–271 (2003).

Yoshinaga N, Shiraishi E, Yamamoto T, Iguchi T, Abe S, Kitano T: Sexually dimorphic expression of a teleost homologue of müllerian inhibiting substance during gonadal sex differentiation in Japanese flounder, *Paralichthys olivaceus*. Biochem Biophys Res Commun 322:508–513 (2004).